

Use of a naturally occurring codon bias for identifying topoisomerase mutations in ciprofloxacin resistant *Escherichia coli* using PCR and future prospects with other bacterial genera: A pilot study*

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ABSTRACT

We developed a novel PCR method aimed at identifying and amplifying native codon sequences of mutation-prone amino acids in DNA gyrase implicated in quinolone resistance using a naturally occurring codon bias in *E. coli* DNA gyrase A.

Keywords: *Escherichia coli* Gyrase A; Codon Bias; Mutation; Ciprofloxacin Resistance; PCR

1. INTRODUCTION

The bacterial DNA gyrase enzyme induces negative supercoils in the DNA during replication and is the target of action for quinolone drugs [1]. *Escherichia coli* (*E. coli*) has 2 sub units, namely gyrase A (*gyrA*) and gyrase B (*gyrB*) that have molecular weights of 97 kDa and 90 kDa respectively [2]. *GyrA* interacts with DNA and cleaves it using its active site tyrosine residue (Tyr122) while *GyrB* has an ATPase active site. Recently, a small angle scattering X-ray study revealed the structure of *GyrA* and *GyrB* bound to DNA in *E. coli* [3]. Mutations in gyrase A (*gyrA*) lead to bacterial resistance to quinolone drugs. Substitution of serine at residue 83 (*ser83*) in gyrase A is the single most important reason for resistance to quinolones [4]. Mutations at *ser83* of *gyrA* is the most frequent in *Escherichia coli* (*E. coli*) clinical isolates [5]. Mutation of *ser83* to leucine (Leu) or tryptophan (Trp) confers high level resistance to quinolones while *ser83* to Alanine (Ala) confers low level resi-

stance [6]. Similarly, aspartate at residue 87 (*asp87*) is another important target for quinolone drugs. Mutation of *asp87* in gyrase A to asparagine (Asp) or valine (Val) is common [7]. But in *E. coli*, the serine codon for primordial genes like ATP synthase and topoisomerase II is highly biased to TCN (where N can be A, T, G or C) than AGY (where Y can be C or T) [8]. Quinolone resistance is on the rise among *E. coli* clinical isolates [9]. Although regular disk diffusion techniques are employed to identify quinolone resistance [10] the molecular basis of quinolone resistance in *E. coli* attributed to mutations in topoisomerase II and presence of *qnr* genes could provide further understanding of the consistency of such resistance patterns.

PCR based detection of mutations that confer resistance, not only saves time when compared to disk diffusion methods, but also helps in identification of the nature of mutations that influence the resistance to antibiotics. Although various techniques for identifying mutations in gene sequences have been employed in microbiology laboratories around the world, they are not time and cost effective. We propose a new type of PCR using primers for amplification of native codon sequence or PAN PCR. By this method, only the native codon sequence is amplified in a PCR reaction while a mutated sequence will not. The naturally occurring codon bias in *E. coli* especially with respect to gyrase A forms the basis of this assay. This method was found to be quite efficient in identifying mutations in *gyrA*. Hence, identification of such codon biased sequences and design of PCR techniques targeting the mutations, not only in gyrase A but also in the target proteins for aminoglycosides, penicillins and macrolides.

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2. MATERIAL AND METHODS

2.1. Bacterial Cultures and Disk Diffusion Experiments

52 non-repetitive *E. coli* strains were isolated from patients at the Department of Microbiology, PSG Institute of Medical Sciences and Research during 2008 and their antibiotic resistance profile for ciprofloxacin, ofloxacin, norfloxacin and nalidixic acid (HiMedia, India) was made using disk diffusion method as per CLSI guidelines. *E. coli* ATCC 25922 was used as a quality control strain for the experiments.

2.2. Primer Design for *gyrA*, *parC* and *qnr*

The primers for control *gyrA* and *parC* were designed as follows: *gyrA*-F, 5'-GACCTTGCGAGAAGAAATTA-CAC-3' (position:7-28); *gyrA*-R, 5'-GATGTTGGTTGC-CATACCTACG-3' (position 546 - 525). These sets of primers were used as internal control for the PAN PCR reactions. For analysis of the presence of the native codon sequence, the forward primer of *gyrA* was used with *gyrA*ser83R, 5'-TCGTGTCATAGACCGCCG-3' (position 265-248) or *gyrA*asp87R (5'-GCGCCATGCG-GACGATCGTGTC-3'). For analyzing plasmid-mediated quinolone resistance, we assessed the presence of *qnr* genes (*qnrA1* to *qnrA6*, *qnrB1* to *qnrB6* and *qnrS1* to *qnrS2*) using primer sequences published by Cattoir and others [11].

2.3. PCR Conditions

All the primers were purchased from Integrated DNA Technologies, USA. For the PCR reaction, the heat lysis method described previously [12] was followed. Briefly, 1 μ L of supernatant containing template DNA was added to 9 μ L of master mix containing 5 pM of forward and 5 pM each of reverse or PAN primers, 1 μ L of 10 \times *Taq* buffer (GeneI, India) and 0.5 units of *Taq* DNA polymerase (GeneI, India).

The steps of PCR were as follows: Initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 52°C for *gyrA* and 47°C for *parC* for 1 minute and 30 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 5 minutes. PCR products were resolved on 1% agarose gels in 0.5 \times TBE buffer and stained with ethidium bromide for visualization.

3. RESULTS

In the disk diffusion method, 43 strains showed resistance to at least one of the four quinolone antibiotics (82.6%) and 9 strains were susceptible to all the four antibiotics. Among these 43 strains, 5 strains showed

resistance only to nalidixic acid but were susceptible to the other three. The remaining 38 strains were resistant to all the four antibiotics (73%).

The resistance patterns observed by disk diffusion method extrapolated on to the PAN PCR results that exhibited specific mutation patterns, except for one strain, which was resistant to quinolones in the disk diffusion method but did not exhibit any mutations in *gyrA*. This could be due to presence of mutations in other genes like *parE* or *aac6'-lb-cr* or efflux pumps [13]. Among the resistant strains, 28 strains (68.4%) showed mutation in *ser83* alone of *gyrA* while 2 strains showed mutation in both *ser83* and *asp87* of *gyrA*. PCR patterns of 4 representative strains are shown in **Figure 1(a)**. One strain (isolate E5) showed mutation in *ser80* of *parC* (**Figure 1(b)**, lane 4A and **Table 1**). Of the remaining 9 resistant strains, 8 of them did not exhibit any mutations but harbored the *qnrS* or *qnrB* genes (4 of them had *qnrS* and the remaining 4 had *qnrB*) (**Figure 1(c)** and **Table 1**).

4. DISCUSSION

Resistance to antibiotics in *E. coli* has become a common feature in developing countries, especially in India, where amoxicillin and ciprofloxacin are the commonly prescribed antibiotics [14]. We developed a new method for detecting such mutations leading to antibiotic resistance, especially quinolones, using PCR based on naturally occurring codon bias and redundancy [8,15]. The efficiency of this method was also verified using some representative nucleotide sequences of *gyrA* from *Escherichia coli* genome maps available in the National Center for Biotechnology Information (NCBI) (**Table 1**). It was interesting to note that almost all of the strains had the same nucleotide sequence from bases 240 to 261 that comprise the *ser83* and *asp87* regions for *gyrA*. This observation is consistent with the codon bias (TCG for serine and GAC for aspartate) that was observed in *E. coli* [13]. Another interesting observation was that *E. coli* strain SMS-3-5 had mutations both at *ser83* and *asp87*. As indicated in **Table 2**, the PAN primers would not amplify the *gyrA* sequence of this strain, if tested. We observed similar mutations (Ser to Leu and Asp to Asn) in the both the strains that possessed a double mutation when subjected to sequencing. This observation is also similar with earlier reports [6,7]. The enteropathogenic *E. coli* (EPEC) reference strain E2348/69 (O127: H6) was found to be ciprofloxacin sensitive in our earlier work [10]. As expected, we observed *gyrA* gene amplification using PAN PCR. Furthermore, the *gyrA* sequence of EPEC (**Table 2**) did not have any mutated sequences for *ser83* or *asp87* which is consistent with the sequence in NCBI which showed no mutations in *ser83* and *asp87* in EPEC's *gyrA* sequence (**Table 2**). But we

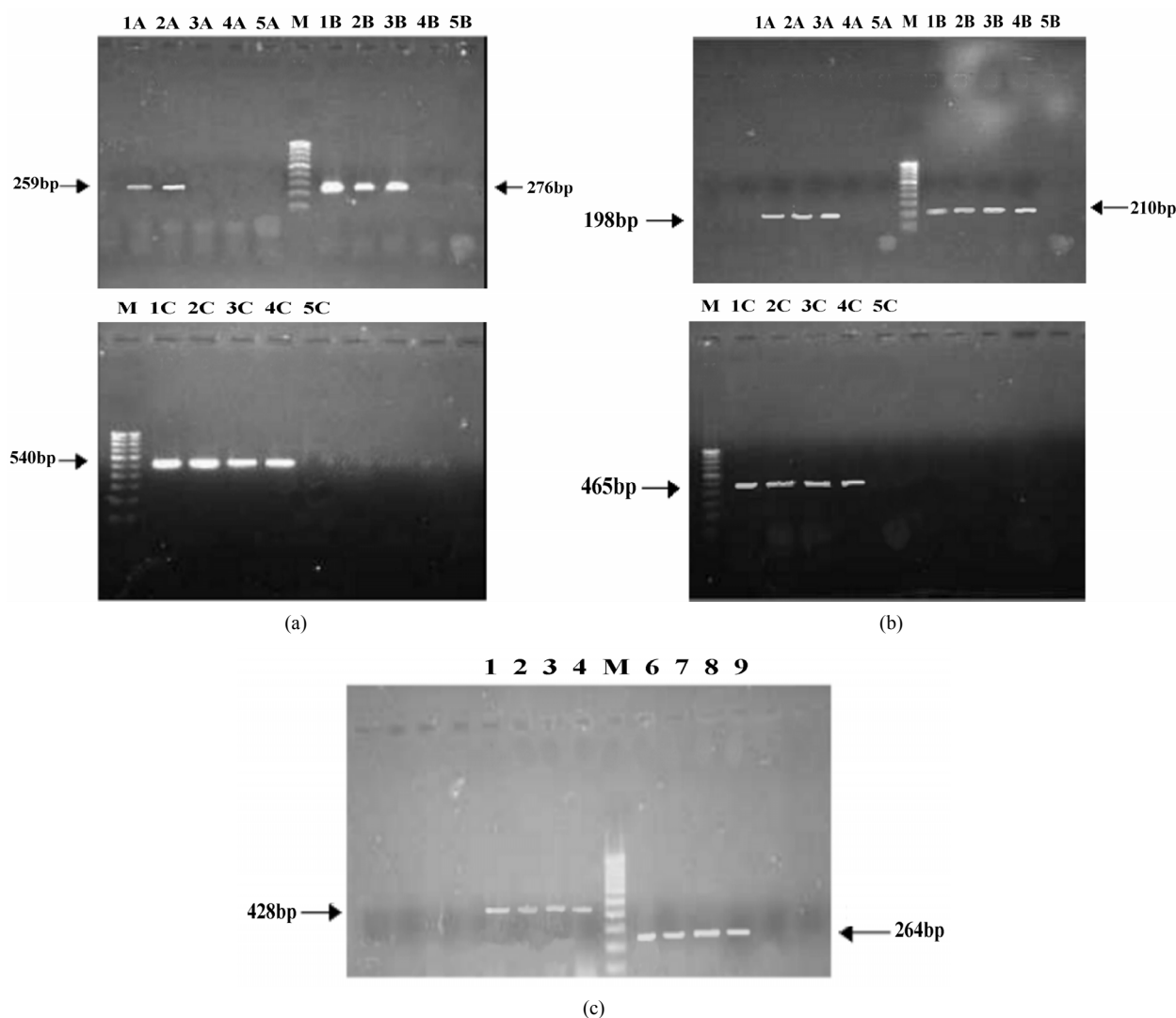


Figure 1. (a) Analysis of *gyrA* mutations by PCR; A: *gyrA* ser83; B: *gyrA* asp87; C: *gyrA* control; M: 100 base pair DNA ladder; 1: Quality control strain *E. coli* ATCC 25922; 2: *E. coli* A1 (No mutation); 3: *E. coli* A3 (mutation in *ser83*); 4: *E. coli* D4(mutation in both *ser83* and *asp87*); 5: PCR negative control; (b) Analysis of *parC* mutations by PCR; A: *parC* ser80; B: *parC* glu84; C: *parC* control; M: 100 base pair DNA ladder; 1: Quality control strain *E. coli* ATCC 25922; 2: *E. coli* A1 (No mutation); 3: *E. coli* A3 (No mutation); 4: *E. coli* E5 (mutation in *parC ser80*); 5: PCR negative control; (c) Analysis of *qnr* genes by PCR; Lanes 1-4: *qnrS* positive strains E6, EHEC, Strain 1310 and Strain 932; Lanes 6-9: *qnrB* positive strains F6, F7, G1 and G2.

observed earlier that many of the EPEC clinical isolates were resistant to quinolones/fluoroquinolones. We subjected them to PCR analysis and found that they harbored mutations predominantly in *ser83* codon.

Laboratories in developing countries seldom have facilities for DNA sequencing analysis. Apart from routine disk diffusion methods, molecular level analyses for the presence or absence of mutations at specific sites that confer resistance to the antibiotic has become imperative. Although DNA sequencing reveals the nature of mutations (*i.e.* Asp-87 to Asn or Ser-83 or Leu or Trp), its importance does not go beyond the mere fact that the substituted amino acid might possibly influence decrease in antibiotic affinity to the target site. On the other hand,

techniques like PAN PCR, though would not reveal the amino acid substitution, can aid in identification of the presence of a mutation. Indeed, the bacterium's natural selection process for codon sets is in fact advantageous, as in this case, to use them in diagnosis. In fact, our further search on *gyrA* sequences in other genera like *Salmonella*, *Shigella* and *Helicobacter* sp. further revealed that the *ser83* and *asp87* is mutated inherently in some of the strains. The PAN PCR would not amplify these sequences too. Hence, this method could also be used to identify quinolone/fluoroquinolone resistance across a wide range of genera. Further targets for antibiotic resistance in these genera could be analyzed for similar conserved codons and hence can serve to develop more

Table 1. Mutation patterns observed in *E. coli* strains. “+” indicates amplification (presence of native sequence) and “-” indicates lack of amplification (presence of mutation) for *gyrA* and *parC* sequences. For *qnr* genes, “+” indicates presence of the gene while “-” indicates absence.

Strain	<i>gyrA ser83</i>	<i>gyrA asp87</i>	<i>parC ser80</i>	<i>parC glu84</i>	<i>qnr</i>
A1	+	+	+	+	-
A2	+	+	+	+	-
A3	-	+	+	+	-
A4	+	+	+	+	-
B1	+	+	+	+	-
B2	+	+	+	+	-
B3	-	+	+	+	-
B5	+	+	+	+	-
B6	+	+	+	+	-
B7	+	+	+	+	-
C1	-	+	+	+	-
C2	+	+	+	+	-
C3	-	+	+	+	-
C4	+	+	+	+	-
C5	-	+	?	?	-
C7	-	+	+	+	-
D1	-	+	+	+	-
D3	-	+	+	+	-
D4	-	-	+	+	-
D5	+	+	+	+	-
D6	-	+	+	+	-
E2	+	+	+	+	-
E3	-	+	+	+	-
E5	+	+	+	+	-
E6	-	+	+	+	+
E7	-	+	+	+	-
F1	-	+	+	+	-
F2	-	+	+	+	-
F3	+	+	+	+	-
F4	+	+	+	+	-
F5	-	+	+	+	-
F6	-	+	+	+	+
F7	-	+	+	+	+
G1	-	+	+	+	+
G2	-	+	+	+	+
G3	+	+	+	+	-
G4	+	+	+	+	-
G5	-	+	+	+	-
G6	-	+	+	+	-
G7	-	+	+	+	-
G8	-	+	+	+	-
WT EPEC	+	+	+	+	-
EPEC C2	+	+	+	+	-
EPEC A5	+	+	+	+	-
EHEC	+	+	+	+	+
587	-	+	+	+	-
1310	-	+	+	+	+
924	-	+	+	+	-
2069	+	+	+	+	-
932	+	+	+	+	+
1632	-	+	+	+	-
3123	-	+	-	+	-
ATCC 25922	+	+	-	+	-

Table 2. Prediction of PAN PCR efficacy with representative *E. coli* pathogens: Nucleotide sequence of *gyrA* of different *Escherichia coli* strains from National Center for Biotechnology Information (NCBI). The NCBI sequence reference number is provided within brackets. Nucleotides 240-261 are represented here. Serine codon (TCG) is at positions 247-249 while Aspartate (GAC) is at positions 259-261. Reverse primers are aligned with putative complementary sequences. Red and blue solid boxes are the primer annealing bases from which DNA polymerase extends the complementary strand from *gyrAser83R* and *gyrAasp87R* respectively. Strain *Escherichia coli* SMS-3-5 exhibits mutations in both *ser83* and *asp87* due to base mismatch between 3' terminal base of primer and the DNA sequence. PAN PCR primers would not amplify this sequence and is indicative of quinolone resistance. The remaining 11 representative pathogenic *E. coli* strains have exact complementary base pairs for *ser83* and *asp87* highlighted as dashed red and blue boxes respectively. PAN PCR primers would amplify these sequences and is indicative of quinolone susceptibility.

5'-----3'	
GGT GAC TCG GCA GTT TAT GAC	<i>Escherichia coli</i> O157:H7 EDL933 (NC_002655.2)
GGT GAC TCG GCG GTT TAT GAC	APEC O1 (NC_008563.1)
GGT GAC TCG GCG GTT TAT GAC	<i>Escherichia coli</i> 536 (NC_008253.1)
GGT GAC TCG GCG GTT TAT GAC	<i>Escherichia coli</i> IA139 (NC_011750.1)
GGT GAC TCG GCG GTT TAT GAC	<i>Escherichia coli</i> ATCC 8739 (NC_010468.1)
GGT GAC TCG GCG GTT TAT GAC	EPEC E2348/69 (NC_011601.1)
GGT GAC TCG GCG GTT TAT GAC	<i>Escherichia coli</i> CFT073 (NC_004431.1)
GGT GAC TCG GCA GTT TAT GAC	<i>Escherichia coli</i> EC4115 (NC_011353.1)
GGT GAC TCG GCG GTT TAT GAC	<i>Escherichia coli</i> E24377A (NC_009801.1)
GGT GAC TCG GCG GTC TAT GAC	<i>Escherichia coli</i> DH10B (NC_010473.1)
GGT GAC TCG GCG GTC TAT GAC	<i>Escherichia coli</i> MG1655 (NC_000913.2)
GGT GAC TCG GCA GTT TAT GAC	<i>Escherichia coli</i> O157:H7 str. Sakai (NC_002695.1)
GGT GAC TTG GCG GTT TAT AAC	<i>Escherichia coli</i> SMS-3-5 (NC_010498.1)
3'-GC CGC CAG ATA CTG TGC T-5' <i>gyrAser83R</i>	
3'-CTG TGC TAG CAG GCG TAC CGC G-5' <i>gyrAasp87R</i>	

APEC: Avian pathogenic *Escherichia coli*; EPEC: Enteropathogenic *Escherichia coli*.

detection methods for multiple antibiotic resistance in bacteria. PAN PCR can also reduce the time required for detection of quinolone resistance and help in preventing the indiscriminate usage of quinolone drugs. Clinically, it can open avenues for exploration of other amino acids in gyrase A that can be targets other than these mutation-prone amino acids for developing anti-bacterials. We [10] and others [16] have explored the same, where we have independently reported the importance of Arg-121 in the QRDR region as a pivotal amino acid for ciprofloxacin binding. A small molecule that was found to be highly potent against enteropathogenic *E. coli* was also found to be targeting Arg-121, apart from interacting with other amino acids like Methionine (Met-120), Alanine (Ala-117) and Aspartate (Asp-82) [10].

To conclude, PAN PCR helps in the identification of mutations, though not substitutions, but nevertheless reveals the need for targeting other regions in gyrase A, not only for quinolones, but for other prospective anti-bacterials. It also encourages researchers to search and target more of such native codon sequences that are conserved within and across many other bacterial genera and the mutation of which, might result in antibiotic resistance or increased virulence.

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ABBREVIATIONS

Ala: Alanine;

Arg: Arginine;

Asp/asp: Aspartate;

DNA: Deoxyribonucleic acid;

E. coli: *Escherichia coli*;

Leu: Leucine;

Met: Methionine;

NCBI: National Center for Biotechnology Information;

PCR: Polymerase Chain Reaction;

Ser/ser: Serine;

Trp: Tryptophan;

Val: Valine.